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Dealcoholized red and white wines decrease oxidative stress associated with inflammation in rats

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In vitro experiments have demonstrated that polyphenols exhibit antioxidant and anti-inflammatory activities. The present study was designed to test whether dealcoholized red (DRW) and white (DWW) wines can decrease the oxidative stress associated with inflammation *in vivo*. Rats were fed for 15 d either a control diet or one supplemented with DRW or DWW. Finally, a granuloma was induced by subcutaneous administration of carrageenan. Although DRW showed higher antioxidant activity *in vitro* than DWW, both wines decreased the number of cells recruited into the granuloma pouch. Malondialdehyde decreased in plasma and inflammatory exudate from rats fed with DRW- and DWW-rich diets. Moreover, the concentration of NO increased in exudate, which correlates with the increase in the citrulline:arginine ratio. Polymorphonuclear leucocytes from the inflammatory exudate of rats fed dealcoholized wines showed decreased superoxide anion ($O_2^{\cdot-}$) production and increased NO production *ex vivo*. This change in NO production resulted from increased expression and activity of inducible NO synthase (EC 1.14.13.39). Moreover, the up regulation of cyclo-oxygenase-2 (EC 1.14.99.1) protein expression observed in rats fed the DRW-rich diet was not related to a direct effect of NO. The present results indicate that the non-alcoholic compounds of wines not only improve antioxidant status in an inflammatory situation, but also limit cell infiltration, possibly through a decrease in $O_2^{\cdot-}$ and an increase in NO production.

Free radicals: Nitric oxide synthase: Polymorphonuclear leucocytes: Polyphenols

Most of the beneficial effects associated with the moderate consumption of red wine are related to polyphenols, although wines contain a broad range of non-polyphenolic compounds. *In vitro* studies have shown that polyphenols have antioxidant[1–4] and anti-inflammatory activities. The anti-inflammatory activities are a consequence of the inhibition of phospholipase A_2 (EC 3.1.1.4)[5], cyclo-oxygenase (COX)-2[6] and 5-lipoxygenase[7] (EC 1.13.11.34). However, evidence from the *in vitro* experiments cannot be extrapolated to *in vivo* settings, since polyphenols are extensively metabolized *in vivo*[8–10], which can affect their activities.

The inflammatory response is characterized by the production of chemical mediators, and the presence of large amounts of exudate, proteins and cells in the inflamed area. Neutrophils are the first blood cells that migrate towards inflammatory lesions and they have the capacity to express NADPH oxidase, inducible NO synthase (iNOS) and COX-2. We have observed, in a carrageenan-induced granuloma model of inflammation, that polymorphonuclear (PMN) leucocytes generate superoxide anion ($O_2^{\cdot-}$) and NO[11] and it has been described that $O_2^{\cdot-}$, by reacting with NO, generates peroxynitrite[12,13]. Thus, preventing the generation of $O_2^{\cdot-}$ by inhibiting the enzymes responsible or scavenging $O_2^{\cdot-}$ or avoiding peroxynitrite formation may attenuate inflammation.

It is likely that low levels of flavonoids and their metabolites exert biological effects such as altered cell signalling and gene expression that will contribute to the modulation of oxidative stress and inflammation. These activities have been more extensively studied *in vitro* than *in vivo*[6,14]. However, wine also contains alcohol that has been reported to enhance lipid peroxidation[15] and can thus mask the effects of the non-alcoholic compounds. Our purpose was to explore the effects of dealcoholized red (DRW) and white (DWW) wines on rats with carrageenan-induced granulomas, because of the wines' absence of alcohol and also their differing polyphenol content. This model allowed us to examine the effects of both dealcoholized wines on oxidative stress and whether such effects would be beneficial in situations of inflammation.

Abbreviations: COX, cyclo-oxygenase; DRW, dealcoholized red wine; DWW, dealcoholized white wine; iNOS, inducible NO synthase; $O_2^{\cdot-}$, superoxide anion; ORAC, oxygen radical absorbance capacity; PMN, polymorphonuclear.

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Materials and methods

Animals and diets

Male Sprague–Dawley rats weighing approximately 125 g were used (Harlan Interfauna Ibérica, Barcelona, Spain). They were housed in temperature-controlled rooms (21–23°C), with 40–60 % humidity, and exposed to a 12 h light–dark cycle.

Rats were randomly divided into three groups of seven rats and fed one of the following semi-purified diets for 15 d: (1) a control diet; (2) a 35 % (v/w) DRW diet; or (3) a 35 % (v/w) DWW diet (Table 1). Chow was prepared weekly[16] and stored at −20°C under vacuum to prevent oxidation and loss of antioxidants. Fresh food and water were provided in excess every day, and food consumption was recorded for every cage. All experimental protocols were reviewed and approved by the Ethical Committee of the University of Barcelona, in accordance with European Community guidelines.

Dealcoholized wines were prepared from common commercial wines available in Spain. Alcohol was removed using a rotary evaporator at a maximum temperature of 30°C. Vacuum was applied progressively to avoid mechanical stress. Evaporated ethanol was replaced by acidulated distilled water and the pH was adjusted to that of the original wine. GC was used to identify any traces of ethanol in dealcoholized wines. We have also evaluated the ascorbic acid and fructose content in wines by fluorimetric[17] and colorimetric techniques (Boehringer Mannheim, Darmstadt, Germany), respectively.

Liquid chromatography–mass spectrometry analysis, total phenol content and antioxidant activity of wines

Wines were diluted in methanol–formic acid 1 % (90:10, v/v) and the resulting solutions were sonicated for 10 min and centrifuged at 1000 ***g*** for 10 min. One sample of the supernatant fraction was filtered through a Millipore 0·2 μm disk and 20 μl was used for analysis.

The chromatographic system for liquid chromatography–MS analysis of wines[18] included an Alliance model 2695 (Waters, Milford, MA, USA) coupled with a diode array detector (model 2996; Waters) and a triple-quadrupole mass spectrometer (model Quattro micro; Micromass, Beverly, MA, USA). The analytical column was a 3·5 μm C_{18} Symmetry 150 × 2·1 mm (Waters). The column was maintained at 30°C. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both with 0·1 % formic acid. The flow rate was set at 200 μl/min. The separation was carried out with a linear solvent gradient program starting at 0 % B and ramping to 20 % B in 30 min and to 60 % in 10 min. The mass spectrometer was equipped with an electrospray source operating both in the positive (anthocyanin analysis) and negative (polyphenols) ion mode. The capillary voltage was set to 3·2 kV, the sampling cone voltage to 40 V, and the source temperature to 130°C. The analysis was carried out in full scan mode in the range 100–1000 atomic mass units, while UV-Vis chromatograms were acquired in the range 200–600 nm. All data were acquired by Masslink 4.0 software (Micromass).

Anthocyanin calibration curves were obtained from stock solutions prepared by dissolving 5 mg standard powder (Extrasynthese, Genay, France) in 5 ml methanol–formic acid 10 % (90:10, v/v). Gallic, *p*-coumaric, ferulic and caffeic acid, catechin, epicatechin, *trans*-resveratrol (Sigma, St Louis, MO, USA), myricetin, quercetin (Extrasynthese) and stock solutions were prepared by dissolving 20 mg standard powder in 20 ml methanol. The working solutions were prepared in the range 1–50 μg/ml.

Total phenols in dealcoholized wines were measured following the Folin–Ciocalteau colorimetric method, and utilizing gallic acid as a standard[19].

The antioxidant potential of dealcoholized wines was determined by using four methods. The reducing power was determined in 150 μl according to the method described by Oyaizu[20], using quercetin as a standard. The oxygen radical absorbance capacity (ORAC) assay was used to elucidate the antioxidant behaviour of dealcoholized wines (dilutions 1:25, v/v) against the peroxyl radicals by using *R*-phycoerythrin as a redox-sensitive fluorescent indicator and 2,2′,-azobis-(2-amidinopropane) dihydrochloride as a chemical peroxyl radical generator[21]. *R*-phycoerythrin fluorescence decline was measured at 37°C as the difference between 5 and 30 min (excitation wavelength 530 nm, emission wavelength 585 nm). The $O_2^{\cdot-}$-scavenging activity was quantified in serial dilution of wines using the hypoxanthine–xanthine oxidase (EC 1.17.3.2) system, whereby the reduction rate of nitroblue tetrazolium to a dark-blue formazan is measured at 540 nm every 1 min for a 20 min period[22]. Maximal velocity was computed to determine the necessary concentration for inducing a 50 % scavenging of the $O_2^{\cdot-}$ produced. NO scavenging was determined according to the method described by Vriesman *et al.*[23] with some modifications. Briefly, NO was chemically generated by dilution of 50 μl 50 mM-$NaNO_2$ (giving 250 nmol NO/l) in 10 ml 0·1 M-H_2SO_4 and 0·1 M-KI (pH 1·0). After 2 min, DRW and DWW were added to a final concentration of 1:100 to 1:1000, with Hb used as the standard. During measurement, the test vial was kept under a stream of N_2. NO concentration was monitored using an Iso-NOP NO sensor (World Precision Instruments, Saratosa, FL, USA). The natural logarithm of the electrode values was plotted *v.* time and the (pseudo) first-order reaction constant for scavenging was calculated.

Table 1. Composition of semi-purified diets (g/kg)*

	Diet		
Component	Control	DRW	DWW
Casein	225	225	225
Potato starch	446	446	446
Sucrose	223	223	223
Cellulose	31	31	31
dl-Methionine	1	1	1
Mineral mix†	14	14	14
Vitamin mix‡	10	10	10
Maize oil	50	50	40
Water	350	0	0
Wine	0	350	350

DRW, dealcoholized red wine; DWW, dealcoholized white wine.
* Pellets were dried at room temperature for 12 h.
† AIN-93M-MX (ICN Pharmaceuticals, Costa Mesa, CA, USA).
‡ AIN-93VX (ICN Pharmaceuticals).

Induction of inflammation and evaluation of inflammatory parameters

After day 15 of the experimental feeding period, a granuloma was induced by subcutaneous administration of 6 ml air, followed 24 h later by 4 ml carrageenan (Hispanoland, Barcelona, Spain) 2 % (w/v) in sterile saline, into the dorsum of rats as previously described[24]. Rats were anaesthetized with isofluorane (Abbott Laboratories, Madrid, Spain) 24 h after carrageenan injection. Blood, extracted by heart puncture, and the inflammatory pouch exudates were harvested following overnight fast using heparin-treated syringes. The pouch cavity was then rinsed with 3 ml saline to recover all cells and the granulomatous tissue extracted and weighed. Blood and exudates were centrifuged at 800 ***g*** and the exudates obtained in the presence of EDTA were filtered through Millipore 10 kDa filters (Millipore, Bedford, MA, USA). After hypo-osmotic lysis of contaminating erythrocytes, PMN cells were re-suspended in PBS (pH 7·4) without Ca^{2+} or Mg^{2+}, washed twice and re-suspended in the same buffer with 2 mM-Ca^{2+} and 0·5 mM-Mg^{2+} and counted[11]. Samples of plasma and exudates were flushed with N_2 and stored at − 80°C.

The osmolarity and protein concentration were evaluated in the supernatant fraction of the exudates using a micro-osmometer (Advanced Instruments, Needham Heights, MA, USA) and the Biuret method, respectively. For PGE_2 measurement, 50 μl supernatant fraction of the exudates were processed through C_{18} solid-phase Sep-Pack cartridges (Waters), previously activated with methanol and acidified water (pH 4·0). Eluates from 5 ml methanol were evaporated under N_2, and the resulting dried residues were re-suspended for PGE_2 immunoassay analysis (Cayman Chemicals, Ann Arbor, MI, USA).

Parameters related to oxidative stress in plasma and supernatant fraction of the exudates

We measured the total phenol and the antioxidant potential, both in plasma and exudate supernatant fractions. For antioxidant potential, we used three methods described elsewhere.

Malondialdehyde was assayed as an endproduct of lipid peroxidation. Malondialdehyde equivalents were measured in 200 μl samples using the thiobarbituric acid-reactive substances method, with tetraethoxypropane as a standard[25]. Thiobarbituric acid-reactive substances were extracted with butanol. The tubes were centrifuged at 450 ***g*** for 10 min and the absorbance of the upper phase was measured at 540 nm.

NO released in plasma or in exudate supernatant fractions is oxidized to nitrites. NO was thus measured in deproteinized samples (100 μl) by 20 % TCA (1:1) at 4°C and centrifuged at 18 600 ***g*** for 15 min. Supernatant fractions (50 μl) were incubated in 5 ml of a solution (10 mM-KI, 10 mM-H_2SO_4) converting nitrites to NO, which was evaluated by the 2·0 mm NO sensor (ISO-NOP). The maximal signal was recorded and compared with a $NaNO_2$ standard.

The relationship between free L-citrulline *v.* free L-arginine was also measured in deproteinized and ultramicrofiltrated exudate supernatant fractions. Amino acid analysis was conducted by cation-exchange chromatography coupled to an auto-analyser (Alpha Plus; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the method of Moore *et al.*[26] with post-column derivatisation with ninhydrin. Lithium citrate buffers were used as eluents. Identification and quantification of amino acids was performed according to the retention time and peak areas, respectively, of standards eluted in the same conditions. Norleucine was used as an internal standard. The detection limit was 1 nmol.

Free radical production by polymorphonuclear leucocytes and inducible nitric oxide synthase and cyclo-oxygenase-2 assays

Cells isolated from inflammatory exudates were used to measure $O_2^{\cdot-}$ and NO production as previously described[11]. The viability of the cells was above 90 %, as assessed by the Trypan blue exclusion test, and 85–90 % of the cells were PMN cells. Less than 1 h elapsed between harvesting the exudates and the start of incubation. PMN cells (1×10^6 cells per tube) were incubated for 1 h in PBS, at a pH of 7·4 with 2 mM-$CaCl_2$, 0·5 mM-$MgCl_2$, and phorbol 12-myristate 13-acetate (100 μg/l).

$O_2^{\cdot-}$ generation was assayed by measuring superoxide dismutase (EC 1.15.1.1)-inhibitable reduction of cytochrome c (0·15 mM; horse heart type VI) in the presence of *N*-imino-ethyl-L-ornithine (0·6 mM; Sigma), an NOS inhibitor.

Nitrites plus nitrates were measured as indicators of NO generation by PMN cells, and assays were performed in the presence of L-arginine (0·6 mM) and 150 international units superoxide dismutase. In addition, *N*-imino-ethyl-L-ornithine (0·6 mM) was used as a negative control for each experiment. Incubation medium (60 μl) was treated with 10 μl nitrate reductase (EC 1.7.1.3) (Cayman Chemicals) followed by Griess reagent.

To evaluate iNOS protein expression (Immun-Star Anti-Rabbit Detection kit; Cayman Chemicals), 10^7 PMN cells were incubated in 500 μl lysis buffer containing 50 mM-tri (hydroxymethyl)-aminomethane-HCl (pH 7·4), 500 μM-EDTA, 500 μM-ethylene-glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid, 7 mM-glutathione, 10 % glycerol (v/v), 20 mM-3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate, 100 mM-dithiothreitol, pepstatin (1 μg/ml), aprotinin (1 μg/ml), leupeptin (1 μg/ml) and 2 mM-phenylmethylsulfonyl fluoride for 30 min at 4°C. Samples were sonicated and boiled at 95°C for 3 min. Protein concentration was determined using the Bradford technique. Homogenate samples (20 μg protein per lane) were submitted to gel electrophoresis and blotted onto a nitrocellulose membrane. Purified murine iNOS (Cayman Chemicals) was also loaded (1 μg) as a positive control, and a pre-stained protein standard was used to monitor transfer efficiency. Membranes were then exposed to a 1:1000 ratio of rabbit anti-human iNOS polyclonal antibody (Cayman Chemicals) and a 1:1000 ratio of rabbit anti-chicken polyclonal γ-tubulin (Sigma) as housekeeping for 1 h. The relative densitometric units with regard to murine iNOS were evaluated.

PMN iNOS activity in rats fed the different diets was measured by quantifying the amount of [^{3}H]L-citrulline formed from [^{3}H]L-arginine in 10^7 cells. [^{3}H]L-citrulline content (Cayman Chemicals) was measured by liquid scintillation counting using a Packard Top-Count instrument (Packard Instrument Company, Meriden, CT, USA). To selectively measure iNOS, a Ca^{2+}-free buffer was used with ethylene-glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid present in a final concentration of 1 mM. Non-enzymic conversion

was determined by using a heat-inactivated (2 min at 100°C) cell suspension containing 10^7 cells. As a negative control for each experiment, 1 mM-N^G-nitro-L-arginine methyl ester-HCl (Sigma), an inhibitor of NOS, was added to the incubation medium for 30 min.

To evaluate COX-2 protein expression, 10^7 PMN cells were incubated in 500 μl of the lysis buffer used for iNOS protein expression for 30 min at 4°C. Samples were treated as for iNOS expression. In addition, purified murine COX-2 was loaded (2 μg) as a positive control and a pre-stained protein standard was used to monitor electrophoresis and transfer efficiency. Membranes were then exposed to a 1:2000 ratio of rabbit anti-murine COX-2 polyclonal antibody (Sigma) and a 1:1000 ratio of rabbit anti-chicken γ-tubulin polyclonal antibody for 1 h and then processed as for iNOS expression.

COX-2 activity in PMN cells was measured by quantifying the amount of PGE_2 that can be formed from arachidonic acid (Cayman Chemicals). Cells (10^7) were incubated in 500 μl buffer (pH 7·4) containing 0·14 M-NaCl, 1·59 mM-$NaH_2PO_4.2H_2O$, 8·8 mM-$Na_2HPO_4.2H_2O$, 2 mM-EDTA, 100 mM-dithiothreitol, leupeptin (1 μg/ml), aprotinin (1 μg/ml) and 2 mM-phenylmethylsulfonyl fluoride for 30 min at 4°C. Samples were sonicated and boiled at 95°C for 3 min. After centrifugation at 14 000 ***g*** for 10 min, protein concentration was evaluated in homogenates. Homogenate samples (10 μl) were incubated in 1 ml 0·1 M-tri(hydroxymethyl)-aminomethane-HCl (pH 8·0), 5 mM-EDTA and 2 mM-phenol, in the presence of haeme group and arachidonic acid. A COX-inhibitor, indomethacin (10 mM), was added for sample control. After 2 min of incubation at 37°C, the reaction was stopped with 50 μl HCl.

Statistical methods

Statistical analysis of the data was performed using SPSS software, version 11·5 (SPSS Inc., Chicago, IL, USA). The results are expressed as mean values with their standard errors. Data were evaluated using either the unpaired Student's *t* test or one-way ANOVA. Homogeneity of variances was tested by the Bartlett's test. The Student–Newman–Keuls multiple comparison test was used to detect differences among groups ($P<0·05$). Correlations between variables were studied by linear regression.

Results

Dealcoholized wines and diets

According to GC, the ethanol content of DRW and DWW was less than 3 g/l. Red wine had a higher concentration of polyphenolic compounds than white (Table 2). The dealcoholization process did not alter the polyphenolic composition of wines. The total polyphenol content was also higher in DRW than in DWW (8·64 and 1·82 mM gallic acid equivalents/M, respectively; $P<0·001$) (Table 3).

The antioxidant activities *in vitro* were lower in DWW than in DRW (Table 3). Hypoxanthine–xanthine oxidase and ORAC were better correlated with total phenol (*r* 0·9378 and *r* 0·9577, respectively; $P<0·01$) than with the reducing power (*r* 0·9015 and *r* 0·9011, respectively; $P<0·05$). The largest difference observed between the two wines was the 12-fold higher NO scavenging activity of DRW (2·72 mmol Hb equivalents/l) *v.* DWW (0·23 mmol Hb equivalents/M) ($P<0·001$) (Table 3).

Table 2. Phenolic compounds in wines (mg/l)*

(Mean values with their standard errors for two replicates from three bottles of wine)

	Red wine		White wine	
Compound	Mean	SE	Mean	SE
Phenolic acids				
Gallic acid	26·7	0·30	3·2	0·03
p-Coumaric acid	6·7	0·05	0·8	0·04
Caffeic acid	2·5	0·02	0·4	0·01
Other phenolic acids	54·0	0·40	4·9	0·06
Flavanols				
Catechin	29·8	0·10	5·3	0·12
Epicatechin	17·7	0·20	1·9	0·06
Flavonols				
Myricetin	1·8	0·03	ND	
Quercetin	51·4	0·06	ND	
Kaempferol	1·8	0·10	ND	
Myricentin glucoside	3·7	0·02	–	
Quercetin glucoside	1·5	0·01	–	
Isorhamnetin glucoside	5·8	0·06	–	
Anthocyanins				
Pelargonidin-3-glucoside	3·7	0·02	–	
Cyanidin-3-glucoside	0·5	0·01	–	
Petunidin-3-glucoside	4·3	0·04	–	
Peonidin-3-glucoside	6·1	0·07	–	
Malvidin-3-glucoside	29·1	0·15	–	
Cyanidin-3-glucoside-malonyl	1·4	0·00	–	
Peonidin-3-glucoside-*p*-coumaroyl	+2·1	0·00	–	
malvidin-3-glucoside-*p*-coumaroyl				
Trans-resveratrol	3·6	0·11	ND	

ND, not detected.
* For details of diets and procedures, see Table 1 and Materials and methods.

Ascorbic acid and fructose content in both dealcoholized wines was of the order of 6 μM and 0·25 g/l. According to the wine maker the concentration of sulfites was 110 and 120 mg/l for the red and white wine, respectively. Feeding rats a wine-rich diet for 15 d had no effect on food or water ingestion, and the body-weight gain in the treated groups was similar to the control group.

Inflammatory parameters in exudates

There were no significant differences in the weight of granuloma between dietary groups. Similar volumes of exudate, osmolarity and protein concentration in supernatant fractions of the exudates were observed in the three groups of rats. However, the number of cells in inflammatory exudates decreased by about 47 % in both groups of treated rats. PGE_2 concentration increased in exudates from DRW-fed rats (Table 4).

Biomarkers of oxidative stress in plasma and supernatant fraction of exudates

Reducing power, ORAC activities and $O_2^{\cdot-}$ were higher in plasma than in exudate supernatant fractions (Table 5). However, the DRW diet significantly increased ORAC (23 %) and the $O_2^{\cdot-}$ scavenging activity (50 % scavenging

Table 3. Total phenols and antioxidant activities of dealcoholized wines†

(Mean values with their standard errors for two replicates from three bottles of dealcoholized wine)

	DRW		DWW	
	Mean	SE	Mean	SE
Total phenols (mM gallic acid equivalents/M)	8·64	0·06	1·82*	0·05
Reducing power (mM quercetin equivalents/M)	4·88	0·25	1·26*	0·07
Hypoxanthine–xanthine oxidase SC_{50} (μl/ml)	17·00	2·00	32·00*	2·00
ORAC (mM trolox equivalents/M)	3·10	0·10	2·20*	0·10
NO scavenging (mM Hb equivalents/M)	2·72	0·06	0·23*	0·02

DRW, dealcoholized red wine; DWW, dealcoholized white wine; SC_{50}, 50 % scavenging concentration; ORAC, oxygen radical absorbance capacity.
* Mean value was significantly different from that of DRW ($P<0·001$; Student's unpaired *t* test).
† For details of diets and procedures, see Table 1 and Materials and methods.

Table 4. Body weight, exudate parameters and granuloma weight after 24 h of carrageenan granuloma induction*

(Mean values with their standard errors for two replicates from seven rats)

	Control		DRW		DWW	
	Mean	SE	Mean	SE	Mean	SE
Body weight (g)	222·0[a]	15·0	207·0[a]	13·0	217·0[a]	14·0
Weight of granuloma (g)	6·3[a]	1·4	4·0[a]	0·7	5·6[a]	1·0
Volume of exudates (ml)	4·3[a]	0·8	4·9[a]	0·5	3·9[a]	0·6
Osmolarity of exudate (mOsmol/l)	303·0[a]	1·0	300·0[a]	3·0	302·0[a]	2·0
Protein concentration in exudate supernatant fractions (g/l)	1·5[a]	0·1	1·5[a]	0·1	1·6[a]	0·1
Number of cells in exudate (× 10^6)	95·0[b]	7·0	50·0[a]	6·0	51·0[a]	4·0
PGE_2 in exudate supernatant fractions (pg/ml)†	66·0[a]	4·0	82·0[b]	4·0	74·0[a]	5·0

DRW, dealcoholized red wine; DWW, dealcoholized white wine.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* For details of diets and procedures, see Table 1 and Materials and methods.
† Two replicates from seven rats.

Table 5. Oxidative stress in plasma and supernatant fraction of exudates after 24 h of carrageenan granuloma induction*

(Mean values with their standard errors for two replicates from seven rats)

	Plasma						Supernatant fraction of exudates					
	Control		DRW		DWW		Control		DRW		DWW	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Total phenols (nM gallic acid equivalents/M)	950[a]	60	1130[b]	30	1080[b]	40	80[a]	30	91[b]	60	83[a]	40
Reducing power (μM quercetin equivalents/M)	111·0[a]	8·0	134·0[ab]	11·0	147·0[b]	11·0	54·0[a]	4·0	76·0[b]	4·0	76·0[b]	5·0
$O_2^{\cdot-}$ scavenging (hypoxanthine–xanthine oxidase SC_{50}) (μl/ml)	45·0[c]	2·0	26·0[a]	1·0	39·0[b]	2·0	96·0[b]	9·0	70·0[a]	5·0	73·0[a]	4·0
ORAC (mM trolox equivalents/M)	2·2[a]	0·1	2·7[b]	0·1	2·7[b]	0·1	1·4[a]	0·1	2·7[b]	0·1	1·7[a]	0·1
TBARS (μM)	6·0[b]	0·2	5·1[a]	0·3	5·1[a]	0·2	27·0[a]	2·0	22·0[b]	2·0	23·0[a]	3·0
NO (μM)	21·0[a]	2·0	26·0[b]	2·0	28·0[b]	2·0	104·0[a]	7·0	144·0[b]	5·0	155·0[b]	12·0

DRW, dealcoholized red wine; DWW, dealcoholized white wine; ORAC, oxygen radical absorbance capacity; TBARS, thiobarbituric acid-reactive substances.
[a,b,c] Mean values for plasma or supernatant fraction of exudates with unlike superscript letters were significantly different ($P<0·05$).
* For details of diets and procedures, see Table 1 and Materials and methods.

concentration decreased by 42 %) in plasma, and the reducing power (41 %), ORAC (93 %) and $O_2^{\cdot-}$ scavenging activity (50 % scavenging concentration decreased by 27 %) in exudate supernatant fractions. Feeding rats a DWW-rich diet increased the reducing power and $O_2^{\cdot-}$-scavenging activities in plasma (32 and 13 %, respectively) and in supernatant fractions of exudates (41 and 24 %, respectively) and the ORAC (23 %) in plasma. Dietary administration of dealcoholized wines

Table 6. Superoxide anion ($O_2^{\cdot-}$) and nitric oxide production by polymorphonuclear cells*
(Mean values with their standard errors for two replicates from seven rats)

	Control		DRW		DWW	
	Mean	SE	Mean	SE	Mean	SE
$O_2^{\cdot-}$ production (nmol/10^6 cells per h)	4·45[b]	0·49	1·95[a]	0·32	2·51[a]	0·39[a]
NO production (nmol/10^6 cells per h)	0·91[a]	0·29[a]	3·17[b]	0·35	2·95[b]	0·30

DRW, dealcoholized red wine; DWW, dealcoholized white wine.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* For details of diets and procedures, see Table 1 and Materials and methods.

significantly decreased thiobarbituric acid-reactive substances in plasma from both groups of dealcoholized wine-fed rats and in the supernatant fraction of exudates from DRW-fed rats (Table 5). Values in exudate supernatant fractions were about 4·5-fold higher than in plasma (6·0 μM in plasma of control rats).

NO concentration was about 5-fold higher in the supernatant fraction of exudates than in plasma, and significantly greater in dealcoholized wine-fed rats (Table 5). The NO levels in exudate supernatant fractions, which are indicative of NO production by inflammatory cells *in situ*, are consistent with the ratios of free citrulline *v.* free arginine found in this compartment (0·76 (SE 0·08), 1·08 (SE 0·12) and 1·34 (SE 0·12) for control, DRW and DWW, respectively), which is equivalent to increases of 43 and 77 % in DRW- and DWW-treated rats, respectively, as compared with rats fed the control diet. No correlation between NO and number of cells in exudates was observed. However, a positive correlation between NO and citrulline:arginine in the supernatant fraction of exudates was detected (r 0·5304; $P=0·0235$).

Free radical production by polymorphonuclear leucocytes and inducible nitric oxide synthase and cyclo-oxygenase-2 assays

The *ex vivo* $O_2^{\cdot-}$ production by PMN leucocytes isolated from the inflammatory exudates decreased by 56 % in DRW- and by 44 % in DWW-treated rats (Table 6), while NO generation increased by 220 and 200 % in DRW- and DWW-fed rats, respectively (Table 6).

iNOS protein expression increased by 47 and 28 % in DRW- and DWW-fed rats, respectively (Fig. 1 (a) and 1 (b)). However, iNOS activity was more enhanced by the administration of dietary wines (67 and 75 % for DRW and DWW, respectively) than was its expression (Fig. 1 (b)).

COX-2 expression (Fig. 2 (a) and 2 (b)) and activity (Fig. 2 (b)) significantly increased only in rats fed the DRW-rich diet (36 and 30 %, respectively).

Discussion

Polyphenols are important non-alcoholic antioxidant compounds in wine. Most studies on their biological activities have focused on the prevention of atherosclerosis or on the effect on macrophages and platelets, and few studies have been performed using PMN leucocytes as cells present in an acute inflammatory stage. The present paper demonstrates that the administration of DRW- and DWW-rich diets to rats modulates the oxidative stress and the inflammatory response in the carrageenan-induced granuloma pouch, used as a model of acute inflammation.

It has been assumed that red wine shows more protective effects than white wine *in vitro* because of its high content of polyphenolic antioxidants. Moreover, wine also contains non-polyphenolic compounds with antioxidant activity, such as ascorbic acid and sulfites[27]. These two compounds were present at similar concentrations in DRW and DWW and, thus, the differences observed in the antioxidant activities of these wines should be attributed to their qualitative and quantitative differences in polyphenols. However, the similarities in plasma and supernatant fraction of inflammatory exudates from the two treated groups support the view that the bioavailable polyphenols[8–10] and some non-polyphenolic compounds such as ascorbic acid and sulfites can be responsible for the low oxidative stress observed *in vivo*. We can rule out the contribution of uric acid generated from fructose *in vivo*[28] as its concentration in white wine was lower than in red wine, and also because rats have uricase. Small differences

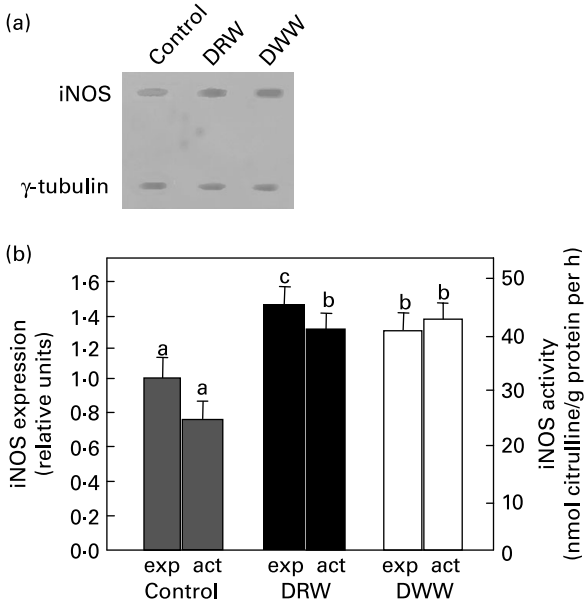

Fig. 1. Inducible NO synthase (iNOS) protein. (a) Representative iNOS expression by Western blot. (b) iNOS expression (exp) and activity (act) in polymorphonuclear cells from the inflammatory exudate of rats fed a control diet, or diets supplemented with dealcoholized red wine (DRW) or dealcoholized white wine (DWW) for 15 d. Values are means with their standard errors represented by vertical bars (two replicates from four rats). [a,b,c]Mean values for exp or act with unlike letters were significantly different ($P<0·05$).

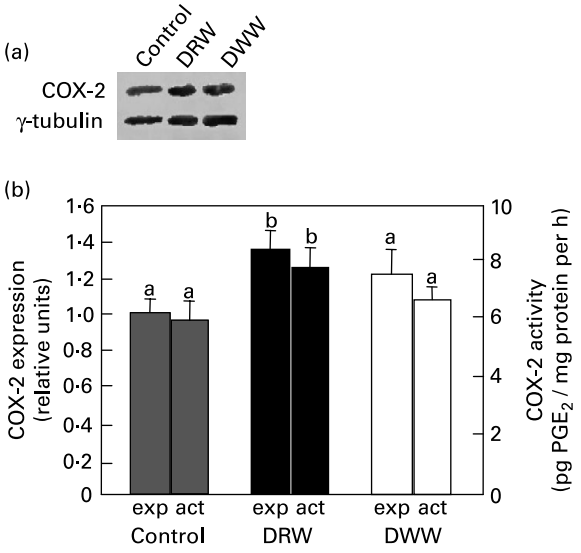

Fig. 2. Cyclo-oxygenase (COX)-2 protein. (a) Representative COX-2 expression by Western blot. (b) COX-2 expression (exp) and activity (act) in polymorphonuclear cells from the inflammatory exudate of rats fed a control diet, or diets supplemented with dealcoholized red wine (DRW) or dealcoholized white wine (DWW). Values are means with their standard errors represented by vertical bars (two replicates from four rats). [a,b] Mean values for exp or act with unlike letters were significantly different ($P<0{\cdot}05$).

in plasma concentration of polyphenols in subjects given red and white wine for 15 d were also observed by Pignatelli *et al.*[29] while the total polyphenolic content of red wine was approximately 7-fold higher.

The effect of DRW and DWW dietary administration on oxidative stress associated with an inflammatory process has also been tested in the present study in PMN cells isolated from inflammatory exudates. When activated, PMN cells release $O_2^{\cdot-}$ (generated by NADPH oxidase and xanthine oxidase) and NO (generated by iNOS). $O_2^{\cdot-}$ has been associated with tissue damage[30] and NO is recognized to be either a protective or a harmful agent[31] depending on the amount produced, the site of production and the presence of other reactive species[32]. In this sense, NO is a good target for $O_2^{\cdot-}$-yielding peroxynitrite[12,13]. Its generation should be deleterious unless an excess of NO remains acting as a protective agent. This latter possibility is confirmed by the high levels of NO in the supernatant fraction of exudates from treated rats and the subsequent decrease in cell number. However, when we studied the *ex vivo* activation of PMN cells in the presence of *N*-imino-ethyl-L-ornithine or superoxide dismutase in the incubation medium, we were able to measure the $O_2^{\cdot-}$ or NO, respectively, in the absence of peroxynitrite generation. In this situation, the two dealcoholized wines had similar effects on both parameters. The decrease in PMN $O_2^{\cdot-}$ release observed *ex vivo* can be linked to a down regulation of NADPH oxidase by NO, as observed in the same model of inflammation[11]. The increase in NO is consistent both with the NO concentration and citrulline:arginine in the supernatant fraction of exudates, which are the result of the *in situ* production of NO by PMN cells recruited into the granuloma pouch, as well as with their iNOS expression and activity. It is difficult to consider polyphenols as only responsible for the increase in NO release. Sharma *et al.*[33] observed that ascorbate regulates NO generation by PMN cells by stabilization of tetrahydrobiopterin, which is a cofactor for iNOS activation. Although we cannot rule out the contribution of ascorbate in iNOS activation, the similar increases in iNOS expression induced by DRW and DWW support the involvement of polyphenols in NO generation. Regarding the similar effects of wines observed in the present paper we have to notice that Whelan *et al.*[34] showed that impaired endothelial function in coronary arterial disease was improved after the ingestion of white and red wine. The decrease in $O_2^{\cdot-}$ generation and the increase in NO by PMN cells and the high levels of NO in the supernatant fraction of the exudates explain the low levels of oxidative stress in plasma and supernatant fraction of the exudates.

Neutrophil migration during an inflammatory response is the result of adhesion on endothelial cells followed by transmigration to the extravascular space and is influenced by a variety of factors, including $O_2^{\cdot-}$ and NO. $O_2^{\cdot-}$ favours the sequestration of leucocytes[14] whereas NO produced by endothelial NOS or by leucocyte-derived iNOS prevents leucocyte recruitment[35–37]. The similar low number of cells in the exudates can be explained by the similar decrease in $O_2^{\cdot-}$ and similar increase in NO by PMN cells after the administration of DRW- and DWW-rich diets. Moreover, neutrophil apoptosis induced by NO may also be involved[37].

In our model of inflammation both $O_2^{\cdot-}$ and NO are generated by activated PMN cells. Up regulation of COX-2 is a key event in inflammation and its relationship to NO is controversial[38,39]. The increase in COX-2 expression and activity in PMN cells and the increase in PGE_2 levels in the supernatant fraction of exudates observed in DRW-treated rats, but not in DWW-treated rats, indicate that under our conditions NO is not critical for COX-2 activation. The paper by Fujimoto *et al.*[39] suggests that PGE_2 production is complex and that H_2O_2, NO and peroxynitrite can have different modulatory effects on COX activities depending on their concentration in cells. The inhibitory effect of polyphenols on COX observed *in vitro*[7] is not observed *in vivo* in the present study and this can be attributed to their metabolism or bioavailability. In this model of acute inflammation, the up regulation of COX-2 could indicate that the DRW was pro-inflammatory. However, the decreased PMN cell number and oxidative stress, the increased NO concentration in supernatant fraction of exudates and the decreased capability of PMN cells to generate $O_2^{\cdot-}$ and the increased NO production can all be considered as anti-inflammatory effects of both DRW- and DWW-rich diets.

During the past decade, we have come to appreciate the prominent role played by inflammation and oxidative stress in several diseases. Our observations indicate that the non-alcoholic compounds of red and white wines are similarly effective *in vivo*. By decreasing $O_2^{\cdot-}$ production and increasing NO production they control cell infiltration and prevent physiopathological processes associated with inflammation. Moreover, NO, by its antioxidant activity, will terminate the lipid radical chain reaction[40,41]. It is thus likely that the antioxidant and anti-inflammatory effects of DRW and DWW *in vivo* can be mainly attributed to polyphenols, although further research is required to elucidate the responsible metabolites.

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